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Methylation Study of a Population Environmentally Exposed to Arsenic in Drinking Water

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Methylation is considered the detoxification pathway for inorganic arsenic (InAs), an established human carcinogen. Urinary speciation analysis is used to assess the distribution of metabolites [monomethylarsonate (MMA), dimethylarsinate (DMA), and unmethylated arsenic (InAs)], as indicators of methylation capacity. We conducted a large biomarker study in northern Chile of a population chronically exposed to high levels of arsenic in drinking water. We report the results of the methylation study, which focused on the effects of exposure and other variables on the percent InAs, MMA, DMA, and the ratio of MMA to DMA in urine. The study consisted of 122 people in a town with arsenic water levels around 600 µg/l and 98 participants in a neighboring town with arsenic levels in water of about 15 µg/l. The corresponding mean urinary arsenic levels were 580 µg/l and 60 µg/l, of which 18.4% and 14.9% were InAs, respectively. The main differences were found for MMA:DMA; exposure, smoking, and being male were associated with higher MMA:DMA, while longer residence, Atacameño ethnicity, and being female were associated with lower MMA:DMA. Together, these variables explained about 30% of the variability in MMA:DMA. Overall, there was no evidence of a threshold for methylation capacity, even at very high exposures, and the interindividual differences were within a much wider range than those attributed to the variables investigated. The differences in percent InAs were small and within the ranges of other studies of background exposure levels. The biological significance of MMA:DMA, which was more than 1.5 times greater in the exposed group, and its relationship to sex, length of exposure, and ethnicity need further investigation because its relevance to health risk is not clear. **Key words:** arsenic, arsenic methylation, arsenic speciation, Chile, water pollution. *Environ Health Perspect* 104:620–628 (1996)

Inorganic arsenic (InAs) is an established human carcinogen. The main sources of human exposure are through inhalation of arsenic dust particles and ingestion from drinking water. Inhalation of arsenic, mainly from occupational exposures, increases the risk of lung cancer, and ingestion of arsenic causes skin cancer, in addition to other characteristic skin alterations such as keratosis and hyperpigmentation (1). More recent evidence indicates that ingested arsenic can also increase the risk of developing lung, bladder, kidney, and liver cancers (2–4). We have estimated that at the present EPA arsenic water standard of 50 µg/l, the internal cancer risks may be comparable to those of environmental tobacco smoke and radon in homes (5).

InAs can be ingested as either arsenite [As(III)] or arsenate [As(V)]. Although As(V) is less toxic, it is reduced biologically to As(III) before methylation, which is considered the detoxification mechanism for InAs (6,7). Two subsequent methylation steps take place: the first one produces monomethylarsonate (MMA), which is then further methylated to dimethylarsinate (DMA). Both MMA and DMA are considered less toxic and bind less to tissues than InAs (6).

It has been postulated that decrease or saturation of methylation capacity may lead

to a threshold for the carcinogenicity of ingested InAs, given that methylation is considered a detoxifying mechanism for InAs (6,8,9). Under this assumption, as exposure increases, one would expect to see an increase in the proportion of InAs, with a corresponding decrease in MMA and DMA.

Several biological media have been used to assess arsenic exposure in humans. Because arsenic is cleared from the blood in a few hours, the arsenic level in blood is not considered a good indicator, especially for low-level exposures (10,11). Although it accumulates in hair and nails, surface arsenic contamination in the form of sorption from external sources also occurs, making arsenic concentrations measured in these samples less accurate for assessing dose (12). Urinary arsenic is generally regarded as the most reliable indicator of recent exposure to InAs and is used as the main biomarker of exposure (11). In the case of ingestion, studies show that around 60–75% of the dose is excreted in the urine (6,7,13).

Although total urinary arsenic has been used to assess InAs exposure, it is important to differentiate InAs and its metabolites from organic forms. In particular, certain types of seafood contain arsenobetaine, a much less toxic, organic form of arsenic

that is quickly excreted in the urine. A recent meal of fish could lead to high measurements of total arsenic (due to arsenobetaine), resulting in an erroneously high evaluation of InAs exposure. Using methods of speciation analysis, InAs, MMA, and DMA can be separated from other arsenic compounds. Currently, the sum of these species in urine constitutes the preferred measure of exposure to InAs, and we henceforth refer to this sum as total arsenic (TotAs). The relative proportions of urinary InAs, MMA, and DMA have been used as a measure of methylation capacity (14).

Previously we presented the results of several studies reporting speciated urinary arsenic measures and showed that population studies do not support the methylation threshold hypothesis (15). The relative percentages of InAs, MMA, and DMA averaged approximately 15–20%, 10–15%, and 60–70%, respectively, across studies, and there was no systematic increase in percent InAs with increasing exposure. In contrast, a large interindividual variability was noted, independent of exposure level. However, the studies were of different populations, exposed to InAs from different sources, and urinary measurements were performed by different methods. In addition, urinary levels did not exceed 400 µg/l, so the question remained as to what happened in more highly exposed groups, such as the Taiwanese populations where high cancer risks have been described. For example, the medium exposure group in Taiwan had

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used drinking water with a weighted average concentration of around 500 µg/l, which would result in urine levels above those published for previous occupational or environmental studies.

A more recent study in the state of Nevada identified individuals drinking well water containing arsenic levels >500 µg/l (mean = 1300 µg/l), with corresponding average urinary measurements of 750 µg/l (16). They were compared to an age- and sex-matched unexposed group in the same area, with mean arsenic water and urine levels of 16 µg/l and 68 µg/l, respectively. This small study ($n = 18$ matched pairs) did not find evidence of a methylation threshold at these high levels; percentages of InAs found for exposed and unexposed groups were similar (19.5% versus 18.6%).

Recently attention has focused on the relative distributions of MMA and DMA in the urine by exposure levels. A study in Mexico found a significant increase in the proportion of MMA relative to DMA among people drinking water with an arsenic concentration of 400 µg/l, compared to a control group at 19 µg/l. Although the proportions of InAs did not differ, the authors postulated that the second methylation step was impaired in the exposed individuals (17). Other investigators have proposed that the MMA:DMA ratio may be a better indicator of the saturation of the methylation pathway than the percent of InAs (18).

The purpose of the study reported here was to investigate arsenic methylation patterns as reflected in urinary metabolites among subjects chronically exposed to high levels of arsenic from drinking water in order to analyze the contribution of exposure, as well as other factors, to methylation capacity. The study was part of a large biomarker investigation conducted in two Chilean towns with different arsenic levels in their water supplies. This paper focuses on a cross-sectional comparison of the relative proportions of urinary arsenic metabolites (InAs, MMA, DMA, and MMA:DMA) with respect to arsenic exposure, as well as with respect to other variables such as sex, smoking, and ethnicity.

Methods

Study population. Study subjects were residents of two towns located in the high Atacama Desert of northern Chile (Fig. 1), and they were mainly of Atacameño ethnic origin. The towns were selected based on their levels of arsenic in drinking water, identified through the results of a previous study (19) and confirmed by subsequent analysis before and during biological sample collection. The water sources derive

from rivers originating in the Andes Mountains and the arsenic content varies depending on the natural geological composition of the rivers' courses (20). San Pedro, with 1600 inhabitants, constituted the high-exposure town. San Pedro has two sources of water supply. Water from the Vilama River is piped to most homes and contains 600–680 µg As/l. The San Pedro River contains about 170 µg As/l and passes through some parts of town as a canal to irrigate nearby farms. Residents without public water supplies use this water for drinking purposes as well. Toconao, with a population of about 360, was selected as the low-exposure, control town, and is located about 40 km from San Pedro. The main source of water in Toconao is the Jerez River, which serves most houses and contains approximately 15 µg As/l.

We recruited study subjects through public announcements and meetings and by door-to-door invitations to participate. Each prospective participant was first interviewed by a local recruiter to ascertain age, smoking status, duration of residence, drinking water source, and interest in participation. They were asked to participate if they were at least 18 years of age and had lived in the town for the last 3 months. As the study progressed, participants from San Pedro and Toconao were selected to be frequency-matched on sex, age, and smoking status. In addition, an effort was made in San Pedro to enroll participants who reported drinking tap water because they were the most highly exposed.

The initial enrollment into the study consisted of 124 participants in San Pedro and 108 in Toconao. Each study subject was interviewed regarding general demographic characteristics, tobacco smoking, and alcohol drinking habits, dietary information including fluid intake, and medical, occupational, and residential histories. It was not possible for interviewers to be blinded with respect to town of residence or to arsenic exposure because interviews were done in each town and the contrasting arsenic levels were widely known. However, because biological samples were identified only by code, urinary arsenic speciation analyses were blinded.

Laboratory analysis. First morning urine samples were obtained by providing participants with precoded, sterilized propylene bottles and written and verbal instructions on how to obtain a clean sample. Samples were collected from participants the next morning and kept frozen in the field laboratory at -20°C until they were transported in dry ice to the University of Washington in Seattle, where they were analyzed for arsenic content.

Urine samples were assayed by hydride generation atomic absorption (HGAA) spectroscopy following a method detailed by Crecelius (21). Briefly, InAs [As(III) and As(V)], MMA, and DMA are reduced to the corresponding arsine in a batch reactor using sodium borohydride. The volatile reduction products (arsine, methyl arsine, and dimethyl arsine, respectively) are removed by sparging with helium. Entrained arsines are concentrated in a chromosorb-filled cryogenic trap at liquid nitrogen temperatures until all arsine-forming arsenic in the sample has reacted. The cryotrap is then allowed to warm, and the collected arsines are separated based on differential volatilization. The detection of the separated volatile arsenic species is accomplished by atomic absorption spectroscopy using a hydrogen microburner combustion cell to convert arsines to elemental arsenic.

Detection limits for InAs, MMA, and DMA were 0.5 µg/l, 1.0 µg/l, and 2.0 µg/l, respectively, with corresponding replicate precisions of 3%, 3%, and 10%. Creatinine measurements were also performed to allow expression of TotAs in relation to urine concentration.

Nine samples had an unusually low proportion of methylated species. Replicates of these samples were tested by spiking in the laboratory with a mixture of known amounts of InAs, MMA, and DMA, which uncovered unusual and strikingly low recoveries of DMA and, to a lesser degree, MMA. It was concluded that for those samples the methylation assay suffered from interference by an as-yet unidentified substance. An alternative method was derived for these anomalous samples, which resulted in more complete recovery of the methylated species, although slightly lower recovery for InAs. Due to differences in methodology and in recovery rates, we chose to omit these samples from the methylation study. We also omitted one other sample from Toconao, an outlier with an unusually

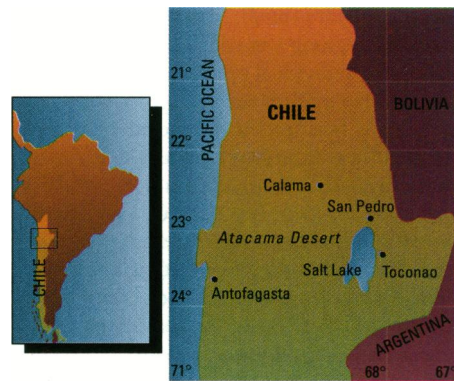


Figure 1. Map of the study area [adapted from Sancha et al. (53)].

high MMA:DMA ratio of 2.4 (several standard deviations away from the mean (0.15) for that town, possibly due to the fact that the TotAs was very low (4.4 µg/l) and species were close to their detection limits.

The recovery rates of urinary arsenic species were calculated by running control samples periodically with urine samples. Because the weighted average recovery rates (over 3 batches of 25, 120, and 19 control samples each) were so close to 100% (98.4%, 100.3%, and 100.1% for InAs, MMA, and DMA, respectively), we did not adjust the assay values of each metabolite.

To rule out any systematic differences between the study subjects whose urine samples were excluded and those that were included, the general characteristics of the two groups were compared. All but one of the excluded subjects were from the low-exposure town, and seven were women. Otherwise, the 10 excluded subjects were comparable in age, ethnicity, years of residence, years of education, smoking, and drinking of alcoholic beverages.

During the study, samples were taken from the various water supplies: tap water from San Pedro and Toconao and water from the San Pedro River, from which some participants drank as well. Water analyses were performed by HGAA according to a procedure similar to that used for urine samples and by a flow-injection analysis method for combined arsine-forming arsenic species (22).

Statistical analysis. We first compared general characteristics of study participants by town, including age, sex, ethnicity, education, length of residence, smoking, and drinking of alcoholic beverages. For the distribution analyses of urinary arsenic metabolites, TotAs (InAs + MMA + DMA), the percentages of InAs, MMA, and DMA, and the ratio MMA:DMA were first calculated by town. We also examined metabolite pattern distribution by reported water source: tap, canal, other, or mixed (e.g., tap water at home, canal water when working near San Pedro canal). The mean and median of each group were derived by first calculating the individual percentage or ratio and then averaging over each category.

TotAs was used as the measure of exposure and was expressed both as a direct measure (µg As/l urine) and as a creatinine-adjusted measure (µg As/g urinary creatinine). The mean urinary arsenic levels were slightly higher when expressed as a function of the urinary concentration of creatinine, a commonly used method to correct for differences in urinary concentrations. However, creatinine excretion can vary considerably by age, sex, lean body mass, diet, and other factors and is not considered a reliable indicator

of urinary dilution, especially for spot samples (23). In some cases creatinine values are used to identify possible outliers (e.g., very dilute or very concentrated urine samples). We examined the distribution of creatinine in our study and found the mean concentration to be 998 mg/g, with 20 of the 220 participants' urine samples either <300 mg/g or >2000 mg/g (approximate values of the lower and upper 5%). We compared the distribution of metabolites including and excluding these samples and found no essential difference. For the regression analyses described below, we compared the results using the unadjusted and adjusted arsenic urine concentrations (µg/l and µg/g creatinine) and found them to be similar. In light of the problems associated with creatinine measurements and the similarities in the results using adjusted or unadjusted values, we decided to use the unadjusted urine measurements expressed as µg As/l urine.

We investigated various factors by univariate comparisons in relation to their possible association with metabolite distribution. Those considered relevant were then entered into multiple regression analyses to assess the contribution of each factor to the dependent variables: percent InAs, percent MMA, percent DMA, and MMA:DMA. Indicator (dummy) variables were used for dichotomous variables such as sex and town of residence. Statistical analyses were computed using Stata 4.0 software (24).

To examine the relationship of ethnicity to arsenic metabolite distribution patterns, we performed a separate analysis on a subsample of the study population restricted to participants of Atacameño and European descent. These two groups constituted most of the study subjects (80%) and excluded those reporting other (specified or unspecified) or unknown ethnicity.

Results

The final study sample consisted of 122 study subjects in San Pedro and 98 in Toconao; general characteristics of the subjects are shown in Table 1. Two subjects, one from each town, were excluded because of incomplete participation (no first morning urine sample). Ten were excluded from the final analysis because of irregularities in the urinary speciation assay as explained above (one from San Pedro, nine from Toconao).

Participants from both towns were generally quite similar, with comparable distributions by sex, age, and educational level (Table 1). The percentages of persons who smoked or drank alcohol were also quite similar. It was common for people to report smoking or drinking occasionally, so we also compared smokers who smoked at

least one cigarette a day to nonsmokers and drinkers who consumed at least one drink a week to nondrinkers [one drink was defined as 12 oz. of beer, 4 oz. of wine, 1 oz. of liquor, or 8 oz. of *aloja* (*aloja* is an alcoholic beverage made from fermented pods of the Algarrobo tree)]. Although the overall proportion of smokers was similar in both towns (28% in San Pedro, 31% in Toconao), both men and women in San Pedro smoked almost 3 times as many cigarettes as those in Toconao (7.2 versus 2.6 cigarettes per day for men, 5.7 versus 2.0 for women). Similarly, the proportion reporting consumption of alcoholic beverages was almost identical in the two towns (57% and 56%); however, men drank about four times more drinks per week than women, and men in San Pedro drank about 75% more than those in Toconao.

The ethnic composition and length of residence varied somewhat between the two towns. Although the proportions of

Table 1. General characteristics of study subjects by town

Characteristic	San Pedro		Toconao	
No. of subjects (%)				
Total	122		98	
Male	69	(57)	52	(53)
Female	53	(43)	46	(47)
Mean age, years (range)				
Male	42.2	(20–74)	42.4	(19–75)
Female	40.1	(18–81)	40.1	(22–70)
Total	41.2	(18–81)	41.3	(19–75)
Mean residence, years (range)	20.0	(0.2–81)	27.9	(0.2–70)
<5	13%		12%	
≥5 to <15	29%		23%	
≥15	58%		65%	
Mean education, years (range)	7.2	(0–19)	7.7	(0–17)
Ethnicity (%)				
Atacameños	91	(75)	71	(72)
Aymara/Mapuche	5	(4)	4	(4)
European	13	(11)	3	(3)
Other	8	(6)	12	(12)
Unknown	5	(4)	8	(8)
Smokers (%)				
All	34	(28)	30	(31)
Male	22	(32)	22	(43)
Female	12	(23)	8	(17)
Smokers of ≥1 cigarette/day (mean cigarettes/day)				
Male	15	(7.2)	13	(2.6)
Female	9	(5.7)	3	(2.0)
Drink any alcohol (%)				
All	70	(57)	55	(56)
Male	50	(72)	43	(83)
Female	20	(38)	12	(26)
Drinkers of ≥1 drink/week (mean drinks/week)				
Male	47	(15.8)	33	(8.8)
Female	13	(2.8)	1	(2.0)

Atacameños and other indigenous groups (Aymara and Mapuche) were quite similar, in San Pedro 11% reported being of European descent, whereas in Toconao only 3% did. Similarly, 10% of San Pedro residents classified themselves as “other or unknown,” compared to 20% in Toconao. The average length of residence was shorter in San Pedro (20 versus 28 years) but was quite long in both, with similar ranges.

The results of arsenic water analyses conducted during the study period for the three main water sources were within the ranges reported previously (19,20). The arsenic concentrations in San Pedro from tap water (from the Vilama River) and from canal water (San Pedro River) were 670 µg/l and 134 µg/l, respectively, while that from the tap water in Toconao was 13 µg/l. The arsenic of the different water sources was all inorganic, but the analytical method we used did not distinguish As(V) from As(III). However, previous analysis of water samples from the same sources found

that practically all of it was As(V) (19).

TotAs and the metabolite distributions by town of residence are shown in Table 2. In San Pedro the mean TotAs was about 10 times that of Toconao (583 µg/l and 61 µg/l, respectively). Four people reported drinking only San Pedro canal water (mean TotAs = 238 µg/l), and 18 reported drink-

ing from mixed sources, although their TotAs levels were similar to those using only tap water (545 µg/l).

The average TotAs concentration of people drinking water from Toconao was somewhat higher than the concentration found in the water, presumably due to the contribution from other sources (e.g., food

Table 2. Levels and relative distributions of urinary arsenic species, by town

Measure	San Pedro (n = 122)				Toconao (n = 98)			
	Mean	SD	Range	Median	Mean	SD	Range	Median
Total As (µg/l)	583	347	61–1,893	482	61	41	6–267	49
Adjusted total (µg As/g creatinine)	637	352	45–2,145	592	68	41	21–306	58
InAs (µg/l)	108	76	5–374	91	8.7	7.2	1.2–41.0	6.6
MMA (µg/l)	91	66	4–329	73	6.1	4.3	0.9–23.0	4.8
DMA (µg/l)	385	234	43–1,225	320	45.8	33.5	3.8–246	39.1
%InAs	18.4	6.1	5.6–38.8	18.2	14.9	5.7	3.6–31.4	14.2
%MMA	15.0	5.5	1.7–30.6	14.8	10.6	4.2	3.1–23.9	9.7
%DMA	66.6	9.9	42.0–92.6	67.1	74.5	8.6	51.2–92.4	75.8
MMA:DMA	0.24	0.12	0.019–0.68	0.23	0.15	0.08	0.034–0.47	0.13

Abbreviations: InAs, inorganic arsenic; MMA, monomethylarsonate; DMA, dimethylarsinate.

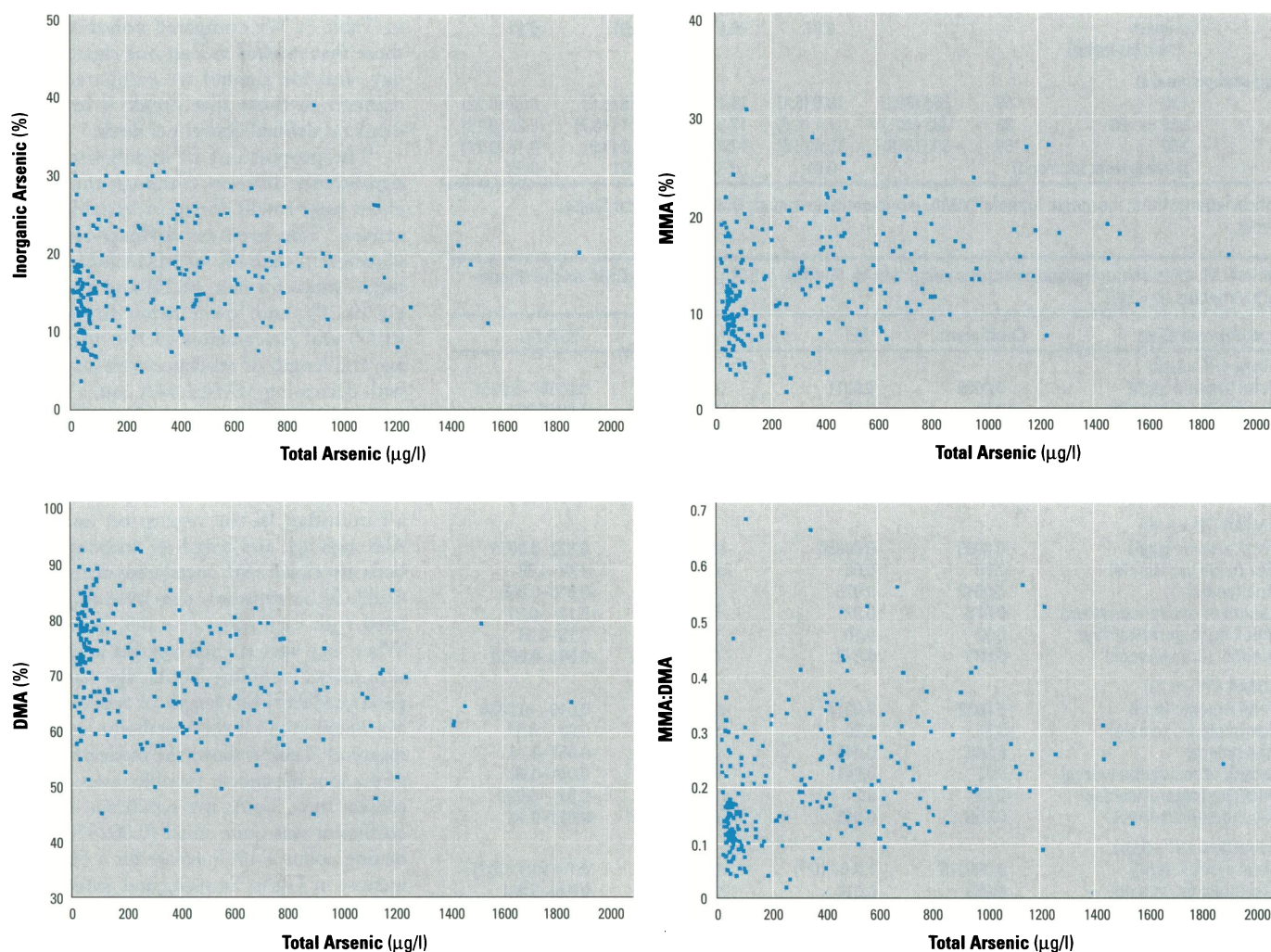


Figure 2. Distribution of percent inorganic arsenic, percent monomethylarsonate (MMA), percent dimethylarsinate (DMA), and MMA:DMA by total urinary arsenic levels for all the study participants from both towns.

Table 3. Urinary arsenic metabolite distribution by various factors

Factor	n	Mean (SD)				
		Total (µg/l)	%InAs	%MMA	%DMA	MMA:DMA
Town						
San Pedro	122	583 (347)	18.4 (6.1)	15.0 (5.5)	66.6 (9.9)	0.24 (0.12)
Toconao	98	61 (41)	14.9 (5.7)	10.6 (4.2)	74.5 (8.6)	0.15 (0.08)
p-value ^a			<0.001	<0.001	<0.001	<0.001
Sex						
Male	121	381 (397)	17.1 (6.0)	14.4 (5.9)	68.5 (10.5)	0.23 (0.13)
Female	99	314 (326)	16.4 (6.3)	11.5 (4.3)	72.0 (9.4)	0.17 (0.09)
p-value ^a			0.43	<0.001	<0.001	<0.001
Ethnicity						
Atacameño	162	371 (390)	16.8 (6.1)	12.6 (4.8)	70.6 (9.4)	0.19 (0.10)
European	16	325 (210)	18.3 (5.6)	17.2 (6.9)	64.5 (11.0)	0.30 (0.16)
p-value ^a			0.34	<0.001	0.015	<0.001
Smoking						
No	156	354 (379)	16.3 (6.2)	12.4 (5.1)	71.3 (9.9)	0.18 (0.10)
≥1/day	41	392 (370)	18.8 (5.4)	15.9 (5.9)	65.2 (10.1)	0.26 (0.14)
p-value ^a			0.018	<0.001	<0.001	<0.001
Alcohol						
No	95	341 (379)	15.4 (5.5)	12.5 (4.8)	72.0 (9.2)	0.18 (0.09)
≥1/week	94	394 (364)	17.8 (6.2)	14.1 (6.0)	68.2 (10.4)	0.22 (0.13)
p-value ^a			0.006	0.048	0.007	0.018
Length of residence (years)						
<5	28	307 (306)	18.6 (5.9)	15.6 (5.9)	65.8 (9.7)	0.25 (0.13)
≥5 to <15	57	411 (428)	17.6 (5.8)	14.3 (5.6)	68.1 (9.3)	0.22 (0.12)
≥15	135	340 (370)	16.1 (6.3)	12.1 (5.0)	71.8 (10.2)	0.18 (0.10)
p-value (test for trend)			0.01	<0.01	<0.01	<0.01
Age groups (years)						
<30	56	355 (365)	18.0 (5.1)	14.4 (5.2)	67.6 (8.9)	0.23 (0.12)
≥30 to <50	96	381 (401)	17.1 (6.9)	12.9 (5.5)	70.1 (10.7)	0.20 (0.12)
≥50	68	303 (318)	15.4 (5.6)	12.3 (5.4)	72.2 (9.9)	0.18 (0.11)
p-value (test for trend)			0.01	0.03	0.01	0.02

Abbreviations: InAs, inorganic arsenic; MMA, monomethylarsonate; DMA, dimethylarsinate.

^at-test.**Table 4.** Multiple linear regression results using %InAs, %MMA, %DMA, and MMA:DMA as the dependent variables (n = 217)

Predictor variables	Coefficient	SE	p-value	95% CI
% InAs ($R^2 = 0.10$)				
Total arsenic (µg/l)	0.0029	0.0011	0.011	0.00067–0.0051
Sex (male vs. female)	0.61	0.85	0.474	-1.06–2.28
Age (years)	-0.45	0.032	0.169	-0.11–0.019
Length of residence (years)	-0.051	0.027	0.061	-0.10–0.023
Smoking (cigarettes/day)	0.10	0.134	0.448	-0.16–0.37
Alcohol (drinks/week)	-0.017	0.030	0.56	-0.075–0.041
% MMA ($R^2 = 0.30$)				
Total arsenic (µg/l)	0.0039	0.00086	<0.001	0.022–0.0056
Sex (male vs. female)	2.76	0.66	<0.001	1.47–4.06
Age (years)	0.0042	0.025	0.867	-0.045–0.053
Length of residence (years)	-0.072	0.021	0.001	-0.11–0.31
Smoking (cigarettes/day)	0.33	0.10	0.002	0.13–0.54
Alcohol (drinks/week)	-0.039	0.022	0.089	-0.084–0.0060
% DMA ($R^2 = 0.23$)				
Total arsenic (µg/l)	-0.0068	0.0017	<0.001	-0.010–0.0034
Sex (male vs. female)	-3.38	1.30	0.010	-5.93–0.82
Age (years)	0.040	0.049	0.415	-0.057–0.14
Length of residence (years)	0.12	0.041	0.003	0.041–0.20
Smoking (cigarettes/day)	-0.43	0.21	0.036	-0.84–0.029
Alcohol (drinks/week)	0.056	0.045	0.219	-0.033–0.14
MMA:DMA ($R^2 = 0.28$)				
Total arsenic (µg/l)	0.000079	1.9 ($\times 10^{-5}$)	<0.001	4.1–11.3 ($\times 10^{-5}$)
Sex (male vs. female)	0.053	0.014	<0.001	0.024–0.081
Age (years)	4.91 ($\times 10^{-7}$)	0.00054	0.999	-0.0011–0.0011
Length of residence (years)	-0.0014	0.00045	<0.002	-0.0023–0.00050
Smoking (cigarettes/day)	0.0081	0.0023	<0.001	0.0037–0.013
Alcohol (drinks/day)	0.00060	0.00050	0.226	-0.0016–0.00038

Abbreviations: InAs, inorganic arsenic; MMA, monomethylarsonate; DMA, dimethylarsinate.

and recent consumption of water in another town). Given the much lower arsenic levels in Toconao water, these factors would contribute a greater proportion of the total than in San Pedro, where water was the overriding arsenic source.

The proportions of InAs and MMA were slightly higher in San Pedro than Toconao (18% versus 15% and 15% versus 11%, respectively), with a corresponding inverse relationship for DMA (67% versus 75%). This resulted in a greater MMA:DMA ratio (0.24 versus 0.15). The association between the distribution of metabolites and TotAs levels across the entire study population is illustrated graphically in Figure 2. No clear, consistent pattern of increase in percent InAs is evident; there is a wide range of interindividual variation. The very slight positive trend for percent MMA and the negative relationship with percent DMA can be synthesized in the ratio of MMA:DMA.

The distribution of arsenic metabolites by factors other than exposure is presented in Table 3. We compared nonsmokers to those that smoked at least one cigarette per day, and for alcohol we compared non-drinkers to those that drank at least one drink (as defined above) per week.

The proportions of metabolites were significantly different (although the differences were small) for most variables presented. The greatest differences were observed in the ratio of MMA:DMA, with higher ratios for men (0.23) and for smokers (0.26), and lower ratios for women (0.17) and Atacameños (0.19). Increasing age and length of residence were associated with decreasing MMA:DMA, and a smaller but positive association was found for alcohol consumption.

Given the effects of these variables on the distribution of arsenic metabolites, they were all included in the regression analyses. Although age and length of residence were both associated with metabolite distribution (Table 3), as expected, they were also correlated with each other ($r = 0.5$, $p < 0.0001$). When they were entered together in a regression model including TotAs, age was not a good predictor, but length of residence was associated with all the metabolite outcome measures. Table 4 shows the models for each of the four dependent variables assessed. For percent InAs, TotAs was significant, but the coefficient was quite small (0.0029%), predicting about a 1% increase for a 500 µg/l increase in TotAs. Smoking and gender were not statistically significant. Length of residence was associated with a decrease in percent InAs, although the magnitude of the coefficient was small (-0.05% per year).

With respect to the methylated metabo-

lites, TotAs, smoking, sex, and length of residence were all contributing factors, whereas alcohol and age showed no clear association. By focusing on the MMA:DMA ratio, the linear regression analysis predicted that a 500 µg/l increase in TotAs would result in a 0.04 change in the ratio, comparable but somewhat lower than the difference found between Toconao and San Pedro (0.15 versus 0.24; Table 2). However, when the regression analysis was restricted to TotAs levels <500 µg/l, the predicted change rose to 0.13 for a 500 µg/l increase, but the coefficient was small and not significant for the model including TotAs >500 µg/l (these models not shown). This suggests that there is an increasing trend in MMA:DMA with increasing exposure up to around 500 µg/l TotAs, which then levels off and is unaffected by further increases in TotAs.

Smoking 10 cigarettes a day had an effect on MMA:DMA (0.08 decrease), as did being male (0.05 decrease). For length of residence, the analysis predicted that a negative change in MMA:DMA of 0.07 would be expected for 50 years of residence (50×-0.0014).

When location rather than TotAs was used as an exposure indicator (Table 5), the other variables remained quite similar, but location contributed to a 0.081 change in MMA:DMA (similar to the difference of 0.09 in the comparison of towns in Table 3). When the analysis was restricted to the 176 individuals of European or Atacameño descent, ethnicity (being Atacameño) proved to be the strongest predictor of the model, with a coefficient of 0.076, but there was little change in the rest of the variables.

Discussion

In this study, the mean percentages of urinary InAs were within the ranges described for two other populations chronically exposed to high levels of arsenic in drinking water, one in the state of Nevada, USA (16), and one in the Region Lagunera, Mexico (17). Percentages of urinary InAs were also within the range of a number of studies including arsenic exposures of background, occupational, environmental, and experimental groups (15).

Although the mean percent InAs was slightly higher in San Pedro than in Toconao, the 3.5% difference was quite small given the difference in mean TotAs of about 500 µg/l between the two towns, and its biological significance is unclear. In the regression analysis the percent InAs was associated with TotAs, but the small magnitude of the coefficient (1.4% increase in percent InAs for a 500 µg/l increase in TotAs) suggested only a weak effect, and

Table 5. Multiple linear regression: additional models

Predictor variables	Coefficient	SE	p-value	95% CI
MMA:DMA, using location (San Pedro vs. Toconao) as exposure measure ($n = 217$; $R^2 = 0.33$)				
Location	0.081	0.014	<0.001	0.054 – 0.11
Sex (male vs. female)	0.060	0.014	<0.001	0.033 – 0.087
Age (years)	-0.00040	0.00053	0.455	-0.0014 – 0.00065
Length of residence (years)	-0.0010	0.00044	0.026	-0.0019 – -0.00012
Smoking (cigarettes/day)	0.0074	0.0022	0.001	0.0030 – 0.012
Alcohol (drinks/week)	-0.00087	0.00048	0.071	-0.0018 – 0.000077
MMA:DMA, restricting analysis to subjects of Atacameño and European ethnic origin ($n = 176$; $R^2 = 0.31$)				
Total arsenic (µg/l)	0.000068	0.000019	<0.001	0.000031 – 0.00010
Sex (male vs. female)	0.044	0.015	0.003	0.016 – 0.073
Age (years)	-0.000081	0.00058	0.891	-0.0012 – 0.0011
Length of residence (years)	-0.0014	0.00046	0.003	-0.0023 – -0.00050
Smoking (cigarettes/day)	0.0048	0.0024	0.049	0.000020 – 0.0094
Alcohol (drinks/week)	-0.00048	0.00046	0.297	-0.0014 – 0.00043
Ethnicity (European vs. Atacameño)	0.076	0.025	0.003	0.027 – 0.12

Abbreviations: InAs, inorganic arsenic; MMA, monomethylarsonate; DMA, dimethylarsinate.

lessened after adjustment for other factors. In contrast, the much larger interindividual variability of about 20% for any given exposure level suggests that factors other than arsenic exposure have an overall greater role in determining the distribution of metabolites. No evidence of a threshold or saturation phenomenon was observed in this population, which included several individuals having TotAs levels well over 1000 µg/l.

With respect to the relative distributions of MMA and DMA, the differences were larger, with an MMA:DMA ratio in San Pedro more than 1.5 times that found in Toconao (0.24 versus 0.15). This is consistent with the results of the only other study, that, to our knowledge, has examined MMA:DMA ratios in similarly contrasting exposure groups (17).

After controlling for exposure, other factors were associated with a higher MMA:DMA ratio, including smoking. The effect of smoking may be related to competition between arsenic and some of the many chemicals found in cigarette smoke for common detoxification pathways or factors. For example, glutathione (GSH) is involved in several steps of InAs metabolism (25,26) and is likely to play a part in the detoxification of several polycyclic aromatic hydrocarbon epoxides generated from cigarette smoke (27). Experimental studies have shown an elevation in GSH in response to arsenic insult (28), and levels of GSH are higher in the bladder tissue of smokers compared to nonsmokers (29). In both cases, the increase in GSH is likely to be a response caused by increased exposure to toxins. On the other hand, pretreatment with GSH depletors impairs arsenic methylation in animal tissue (30–32). While a higher MMA:DMA ratio suggests modulation of the second methylation step, and

the animal studies have suggested that GSH depletion mainly affects the first methylation reaction, these inconsistencies may be due to differences in arsenic methylation that exist between animals and humans. Alternatively, the detoxification of arsenic and tobacco carcinogens may compete for other necessary substrates; for example, binding sites in nonspecific proteins as described for arsenic (33), and/or enzyme systems such as glutathione or methyl transferases.

The sex-related difference in the relative proportions of MMA and DMA was also reported in the recent study in Mexico (34), but there is no clear explanation for this finding. Although women appear to be better arsenic methylators than men, the relevance of this difference to arsenic-related health effects is unknown. Another example of a gender difference in the handling of arsenic comes from an *in vitro* study in the arsenical area of Mexico that found that impaired proliferation was greater in lymphocytes from arsenic-exposed women than from exposed men (35).

Length of residence showed a positive effect on the distribution of all the metabolites, but age did not affect distribution when duration of residence was controlled for. This suggests that an adaptation response may provide a slight improvement in methylation ability.

Ethnicity was a predictive factor: Atacameños had a lower MMA:DMA ratio compared to subjects of European descent. The level of exposure in San Pedro is similar to that of the medium exposure group in Taiwan, where overt signs of arsenicism such as keratosis, hyperpigmentation, and skin cancer were apparent. A previous study found that residents of San Pedro had an increased prevalence of white spots on their skin compared to other towns in the area

with lower water arsenic levels, but more serious skin alterations were not observed (19). The Atacameño people have lived in the region for 11,000 years (36), and studies of mummies buried in the area up to 3000 years ago have revealed high arsenic concentrations in their internal organs (37). All other known populations exposed to high arsenic drinking water have been exposed for much shorter time periods: the Taiwanese for about 70 years (4), the Chilean population of Antofagasta (mainly of Spanish descent) for about 15 years (38), the Argentine population for less than 150 years (39), and the more recently described residents of West Bengal in India for about 30 years (40). All but the Atacameños have shown characteristic keratosis and skin cancer. The question remains whether some characteristic of the Atacameño skin renders it inherently more resistant to these alterations, or whether some other evolutionary adaptation over thousands of years has made Atacameños less susceptible to arsenic, and if so, whether this protective mechanism is related to methylation capacity.

A possible hypothesis that could explain such an adaptation relates to Chagas disease, endemic in many parts of South America including the Atacama Desert. The infection is transmitted by the *vinchuca* bug, a vector of the parasite *Trypanosoma cruzi* (41), of the same family as the African trypanosome that causes sleeping sickness, which was traditionally treated with arsenical drugs. Arsenic inhibits trypanothione disulfide reductase, a GSH equivalent in African trypanosomes necessary for their survival (42). If the South American trypanosome has a similar enzymatic system, it is plausible that over time the Atacameños developed a resistance to arsenic, as it protected against the consequences of Chagas infection [which includes reproductive effects such as congenital abnormalities, abortion, and stillbirths (41)], leading to a selective survival of those more capable of tolerating the toxic effects of arsenic.

Most of the findings in this study are related to variations in the MMA:DMA ratio, and the significance of these differences by factors such as arsenic exposure, sex, ethnicity, and smoking is not clear and needs further investigation. Experimental studies of rat liver cytosol indicate that for high exposures, InAs inhibits the second methylation step, leading to an accumulation of MMA (32), which could partly account for the MMA:DMA differences by exposure levels. One possible explanation is the binding of InAs to a dimethyltransferase involved in the second methylation step (26).

Given the known relative genotoxicities of InAs, MMA, and DMA (roughly

3000:2:1) (43) and the small change in percent InAs across exposure levels, it is unclear how an increased concentration of MMA relative to DMA would contribute significantly to an increased risk of arsenic-induced health effects. Although it has been suggested that during methylation a highly reactive intermediate form of MMA may be formed (26), the existence of this hypothetical chemical has not been demonstrated. Laboratory studies *in vitro* (43,44) and in animals (45) show that although MMA appears to be twice as potent as DMA in genotoxicity and cytotoxicity tests, they are both weak and far less toxic than the inorganic forms. On the other hand, it has also been postulated that an increase in DMA may be more deleterious than previously thought. *In vitro* studies found that DMA can cause chromosomal damage (46,47); similar studies were not reported for MMA. A recent report of the study in Mexico indicated that exposed individuals with skin alterations had a higher MMA:DMA ratio than exposed persons without skin effects (34). However, the exposure magnitude of the two groups was not reported, and the possibility that overall higher exposures to InAs or longer duration of exposure could account for the signs of arsenicism cannot be excluded.

A new hypothesis proposes that competition for methyl groups between arsenic metabolism and DNA methylation could lead to DNA hypomethylation, which has been associated with the changes in gene transcription found in epithelial cancers (48). For this competition to occur, there would have to be a limited availability of methyl groups. Animal studies show that the bioavailability of *S*-adenosylmethionine, the source of methyl groups for arsenic methylation, is not a limiting factor for methylation under normal *in vivo* conditions (31), but it has been suggested that high arsenic exposure may cause the demand for methyl groups to exceed the supply, particularly for individuals with a diet poor in methionine (18). However, it was estimated that exposures to an arsenic concentration of 1800 µg/l in drinking water would require only 1.5% of a person's daily dietary intake of methyl source for arsenic methylation (49), making it unlikely that the supply of methyl groups would be exceeded. Nevertheless, there may be a limited supply of methyltransferases or other chemicals involved in both arsenic and DNA methylation.

The large interindividual variability in methylation ability, as well as the possible ethnic differences, may be due to genetic polymorphisms associated with methylating enzyme activity. Inheritance is a major fac-

tor in individual variation in several methyltransferases involved in the biotransformation of many drugs (50), and the susceptibility to some occupational exposures appears to be associated with differences in detoxification enzymes such as *N*-acetyltransferase and aryl hydrocarbon hydroxylase (51). Genetic polymorphisms of the still-uncharacterized arsenic methylation enzymes may help explain the interindividual variation. Similar genetic differences may exist in arsenic-specific binding proteins, which are thought to decrease the toxicity of InAs by decreasing its tissue availability until it can be methylated (33).

Alternatively, because GSH is involved in arsenic metabolism, genotypic differences in the activity of glutathione transferases (GSTs), which catalyze the conjugation of GSH to a variety of carcinogens and are part of the protective mechanism against cancer caused by environmental carcinogens (52), may affect methylation ability. For example, the null genotype for one of these enzymes, GSTM1, varies from 30% to 70% depending on ethnicity (27).

In conclusion, the factors investigated did not contribute to biologically meaningful differences in the percentage of InAs in urine. The large biomarker study presented here in a chronically highly exposed population indicates that, at high levels of arsenic exposure, there is no evidence of a plateau saturation effect and that variations in methylation capacity, at least as reflected by urinary metabolites, can be quite large. Arsenic exposure level and duration, sex, smoking, and ethnicity were associated with differences in the MMA:DMA ratio, together explaining about 30% of the total variation. It is possible that several genetic polymorphisms operating in the mechanisms of arsenic detoxification play a role in determining methylation capacity, along with other exogenous factors such as diet and other concurrent exposures. The significance of the differences in MMA:DMA ratio in relation to arsenic exposure and to sex and smoking warrants further study, since at present there is no clear evidence of their relevance to health risks. In general, given the current gaps in understanding the mechanisms of arsenic detoxification, several lines of research need to be continued and pursued to confirm or reject some of the more recently proposed hypotheses.

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